Detection and Functional Analyses of Plant Hormones in Apicomplexan Parasite

Detection and Functional Analyses of Plant Hormones in Apicomplexan Parasite

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1. ABSTRACT

Malaria is an important disease which affects 200 million persons and leads approximately 600,000 deaths per year. Malaria parasites *Plasmodium* spp. belong to a phylum Apicomplexa, and currently *Toxoplasma gondii*, another apicomplexan was revealed to produce abscisic acid and cytokinins which are kinds of plant hormones. Although 7 plant hormones are specified in land plants, only 2 of them were identified in *T. gondii* and none in *Plasmodium* spp. In this study, I examined whether other hormones are present in *T. gondii, P. berghei* and *Eimeria tenella* which is a pathogen of chickens. In addition, three major linages of *T. gondii,* RH, ME49 and CTG were separately applied to the analyses to understand the strain-dependent differences. Since the three linages of *T. gondii* are known to be different in pathogenicity, we also discussed the influences of plant hormones to the pathogenicity in *T. gondii*.

Plant hormones were extracted and applied to the UPLC-ESI-qMS/MS system. As a result, abscisic acid, cytokinins, auxin, gibellerin, jasmonic acid and salicylic acid were detected from 3 species. Among them salicylic acid is the most dominant plant hormone that accounts more than 90% of whole plant hormones detected. Cytokinins are divided into 4 active forms; *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z), isopentenyladenine (iP) and dihydrozeatin (dZ). *T. gondii* and *E. tenella* produced both iP and *t*Z, but *P. berghei* only produced iP, probably reflected the differences of species. All 3 species did not possess *c*Z and dZ.

T. gondii has little genetic diversity and it is known that the major lineages can be classified into three clones. Since it has been shown experimentally that these clones differ in pathogenicity for mouse, I discussed the relationship between plant hormones and pathogenicity. From the result, especially, the distribution of the kinds of cytokinins

differed, and it could observe the relationship between the abundance of *t*Z and the virulence. As for *T. gondii*, *t*Z induces the progression of the cell cycle and it also increases the multiplication rate. It also suggests that the relationship between the presence of cytokinins and pathogenicity.

As salicylic acid (SA) presented the most abundantly, I decided to investigate further. Addition of SA did not influence the proliferation of *P. falciparum in vitro*, even 100 μM of SA was added. I tried to detect the orthologues of SA-synthesis genes from the information of known isochorismate synthase (ICS) and benzoic acid (BA) pathways, but they were not identified in any apicomplexan genome databases. Then, I tried to generate a SA-lacking parasite by the transfection of *nahG*, which encodes a SA-degradating enzyme of *Pseudomonas* sp. The concentration of SA decreased approximately at the half comparing to the control infection, which was transfected with *gfp*. The *nahG*-mutant significantly decreased the concentration of parasite-synthesized prostaglandin E_2 that potentially modulates host immunity as an adaptive evolution of *Plasmodium* parasites.

To investigate the function of SA and prostaglandin E_2 on the host immunity, we established *P. berghei* ANKA mutants expressing *nahG,* and confirmed a reduction of plasma prostaglandin E_2. C57BL/6 mice infected with the *nahG*-transfectants developed symptoms of cerebral malaria as assessed by Evans-blue leakage and brain histological observation. The *nahG*-transfectant also significantly increased the mortality rate of mice. Prostaglandin E_2 generally reduced the brain symptom, by induction of T helper-2 cytokines. As expected, T helper-1 cytokines including interferon-γ and interleukin-2 were significantly elevated by infection with the *nahG*-transfectant. Thus, SA of *Plasmodium* spp., which might be a new pathogenic

factor of this threatening parasite, can modulate immune functions via parasite-produced prostaglandin E_2.

2. GENERAL INTRODUCTION

Endosymbiosis and Evolution

Endosymbiosis, an event that turn different species into one, is clearly a dynamic shortcut of evolution (Sagan 1967). The process allows organisms to implant a set of metabolisms, signalings, etc. Mitochondria and chloroplasts are believed to be derived from the ancient endosymbiotic α-proteobacterium and cyanobacterium, respectively (Sagan 1967; McFadden 2001). As results of these events, major energy metabolisms such as aerobic respiration and beta-oxidation of eukaryotes take place inside mitochondria, and carbon fixation of land plants takes in the chloroplasts. Together with these major reactions, isoprenoids are synthesized through the methyl-erythritol phosphate pathway (MEP pathway) of chloroplasts in land plants (Rohmer 1999). The substrates of MEP pathway are pyruvic acid and glyceraldehyde 3-phosphate, and this pathway finally synthesizes isopentenyl diphosphate or dimethylallyl pyrophosphate. In contrast, the synthetic pathway of isoprenoids in animals is called as mevalonate pathway, where synthesis starts with acetyl-CoA. Isoprenoids are essential molecules as precursors of steroids, cholesterols and prenylation of proteins, synthesized by the cytosolic mevalonate pathway in animals, and also involved in variety of plant life: poisonous compounds, vitamins, chlorophylls, and plant hormones (Holstein & Hohl 2004; Lichtenthaler *et al*. 1997; Siperstein1984).

Two hundreds millions of persons are affected by malaria and it leads approximately 600,000 deaths per year (WHO 2013). It is counted as one of three major infectious diseases in the world. This disease is caused by *Plasmodium* spp., eukaryotic parasitic microorganisms which infect to red blood cells of vertebrates. Malaria

parasites belong to a phylum Apicomplexa. The major characteristic of this group is the presence of an organelle 'apicoplast' which is a plastid originated by the secondary symbiosis (Ralph *et al*. 2004). This endosymbiosis might alter the metabolism of the recipient as plant-like: currently *Toxoplasma gondii*, another apicomplexan was revealed to produce the plant hormone abscisic acid (ABA), strongly supports the theory (Nagamune *et al*. 2008). Also, *T gondii* is known to produce cytokinin (CK), other plant hormone, suggests the conservation of plant hormone-regulated signaling (Andrabi *et al*. unpublished data). Only 2 of known plant hormone species have been identified in *T. gondii* and none in *Plasmodium* spp., despite their potential importance.

Plant Hormone (Phytohormone)

Plant hormones, also called phytohormones, are small molecules that regulate the cell division, differentiation, response to both physical and biological stresses, and work at extremely low concentrations. Different from animal hormones, there is no specific organ to produce plant hormone, instead every plant cell is able to produce and secret them. The five major plant hormones are ABA, CKs, ethylene, gibberellin (GA), and auxins. In addition, salicylic acid (SA), jasmonates, brassinosteroids, and plant peptide hormones were recently noticed as plant hormones (Chow & MuCourt 2006). Some of them are released into atmosphere and transfer information to neighbor individuals (Farmer & Ryan 1980). The receptors of at least some out of plant hormones were identified as F-box related proteins, and increase the ubiquitin-dependent digestion of transcriptional repressor, hence increase the transcription of plant hormone-related gene cluster (Santner & Estelle 2010; Dharmasiri *et al*. 2005). SA is known to increase at the site of infection in land plants, and transmits the signal to the whole body (Raskin

1992). Although SA activity to deliver signals fit the definition of plant hormone, the accumulating concentration being extremely higher than other plant hormones poses a unique characteristic.

Apicomplexa

Apicomplexa consists of over 4,000 species, and most of them are obligately parasitic (Levine 1988). Apicomplexa is currently divided into 4 major groups: hemosporidia, coccidia, crypto-gregaline and piroplasmia (Kuo *et al.* 2008). *Plasmodium* spp. are infectious to erythrocytes thus classified as hemosporidia. Coccidia is another large group of this phylum. *Toxoplasma gondii*, a famous human and animal parasite, and *Eimeria* spp., major pathogen of veterinary field, are well-studied species in this group. The life cycle of this phylum basically consists of 3 stages: merogony, gamogony and sporogony. Merogony, also known as schizogony, is an asexual stage within the host, where parasite proliferates rapidly. Gamogony is a stage where parasite differentiates into male and female gametes. These gametes are fused and formed diploid zygote. In this stage, genetic recombination takes place. Sporogony is a stage after fertilization, where parasites perform meiosis once. Resulting haploid parasite, called sporozoite, causes asexual production again. The lifecycles of *Plasmodium* spp., *Eimeria* spp. and *T. gondii* are shown in Fig. 1.

T. gondii infects via oral or vertical route, and tissue cyst/oocyst are responsible for the former. Although *T. gondii* infection is mostly subclinical, it is estimated to affect one third of the world population (Tenter *et al*. 2000). The infection is long-lasting because the parasite spreads to the whole body, forms tissue cyst which is hard to be cleared, and it develops a clinical manifestation once the host immunity is

weaken by HIV/AIDS or by the use of antitumor drugs (Dubey 1998). Another route of infection is the vertical transmission, between mother and child. Recent years the risk of the congenital toxoplasmosis has been paid an attention in Japan, and the importance of this parasite in public health is greatly increasing.

Eimeria spp. are the causative agents of the animal coccidian diseases, and forms the largest group with 1,000 named species (Long 1982). In contrast to *T. gondii*, eimerian infection is strictly limited to the gastrointestinal tract of the host in most species. Therefore the main clinical symptom is diarrhea, bleeding in intestine, or others related to the digestive organ (Hammond & Long 1973). Since the lifecycle is homoxenous, the infectious stage is environmental oocyst. Oocyst endures chemically severe conditions including many kinds of disinfectants and antibiotics, and persists several months in humid condition (Belli *et al*. 2006). Considering with the problem of recently increasing population of drug-resistant parasites, the development of alternative drugs is highly required.

Aim of the study

The aim of my study is to draw the whole map of signaling driven by plant hormones in apicompleans, and to find its potential role in infection. Specially, the influence of hormones in parasites growth, the induced chemicals which potentially modulates the host immunity, and the actual pathogenicity and immune response of the host animals are discussed.

4. INFLUENCE OF SALICYLIC ACID TO *P. FALCIPARUM IN VITRO*

Introduction

I detected extremely high concentration of SA from *Plasmodium, Toxoplasma* and *Eimeria* cell lysates. However, the addition of SA to the culture of the parasite showed no effect. Further studies are required to understand the function of this accumulating plant hormone.

SA originally regulates the pathogen-resistance system of land plants termed systemic acquired resistance (Ryals *et al.* 1996). Pathogen-induced SA is delivered to the whole plant to up-regulate the expression of pathogenesis-related (PR) genes (Ryals *et al*. 1996). Recently, the receptors for SA were identified in *Arabidopsis thaliana* and named as nonexpresser of PR genes (NPR) 3 and 4 (Fu *et al.* 2012). NPR3 and 4 are adaptors of Cullin 3 ubiquitin E3 ligase that degrades the transcription cofactor, NPR1. Namely, SA is a positive transcriptional factor of land plants.

SA is synthesized by two different pathways. The major pathway is the benzoic acid (BA) pathway (Lee *et al.* 1995). The start molecule is L-phenylalanine, and the enzymes, phenylalanine ammonia lyase and BA 2-hydroxylase, catalyze its conversion to SA (Lee *et al.* 1995). Another pathway is the isochorismate synthase (ICS) pathway (Métraux 2002), which is thought to be dominant in *A. thaliana*, and ICS is the key enzyme of this catalysis. I tried to identify the corresponding genes of BA and ICS pathways, but none was identified from the apicomplexan genome database *in silico* as described above. Therefore I tried to establish the SA deficient parasite by transfecting a bacterial SA degrading enzyme, *nahG*.

Because SA and its acetylated derivative, acetylsalicylic acid, inhibit

cyclooxygenase (COX), a prostaglandin (PG) synthetic enzyme in animals, it is used as non-steroidal anti-inflammatory drugs (NSAIDs) (Boynton *et al*. 1988). PGE_2 is the main active molecule acting on the thermoregulatory center to cause fever and pain (Lazarus *et al*. 2007). It might also suppress the host immunity by switching cytokines to a T helper-2 (Th2) phenotype (Kalinski 2012). Interestingly, Kubata *et al.* (1998) demonstrated production of prostanoids in the *P. falciparum* lysates and culture supernatants *in vitro. P. falciparum* releases PGD_2, E_2, and $F_2\alpha$ into the infecting milieu. This is quite interesting because the parasite actively modulates the host immunity by the use of the host signal molecules. Furthermore, the PG synthesizing enzyme of *Plasmodium*, whose protein or gene has not been identified, is biochemically resistant to members of NSAIDs including indomethacin and acetylsalicylic acid (Kubata *et al.* 1998), suggesting the presence of complex interactions among SA, host and parasite PGs. In this chapter, I analyzed the role of *Plasmodium* SA *in vitro*.

Materials & Methods

Parasites

P. falciparum strain 3D7 was provided by The Malaria Research and Reference Reagent Resource Center (MR4), and cultivated *in vitro* as described previously (Trager & Jensen 1976). Human red blood cells (RBC) and serum were provided by the Japan Red Cross.

Addition of salicylic acid to *P. falciparum* culture *in vitro*

After twice synchronization with 5% (w/v) D-sorbitol in Milli-Q water (Merck KGaA, Darmstadt, Germany) as described (Kubata *et al.* 1998), *P. falciparum* was

adjusted to 0.1% parasitemia, cultivated with 100 μM of SA continuously. The same volume of solvent was added to the control (0 μM of SA) group. Parasitemia were observed every 24 hours by methanol-fixed thin blood smear stained with 10% Giemsa solution.

Establishment of salicylic acid-deficient parasites

P. falciparum was transfected with the SA degrading gene, *nahG*, by episomal expression system as reported previously (Zhu *et al*. 2013). Briefly, *nahG* was driven by *P. falciparum* chloroquine resistant transporter gene 5' UTR (pCRT) and *P. berghei* dihydrofolate reductase gene 3'UTR (PbDT), fused into attR4-attR3 site of the destination vector pCHD43 (II) (Sakura *et al.* 2013), and transfected by electroporation. The control parasite line transfected with *gfp* was also made with the same way. The expression of NahG was confirmed by Western blotting with rabbit anti-cMyc antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-HSP-90 antibody as control (Sigma Aldrich, St. Louis, MO, USA).

Growth and cell cycle of salicylic acid-deficient parasites

The *nahG*-expressing *P. falciparum* was monitored its growth and cell cycle progression, and the results were compared to those of the control (transfected with *gfp*). Experimental procedure was identical to the SA-addition experiment. Cell cycle was also observed by thin blood smear stained with 10% Giemsa solution, and the parasite stage was divided into 4 stages: ring, trophozoite, shizont and gametocyte.

SA and prostaglandin quantification

SA quantification of *P. falciparum* was performed biochemically by a commercial salicylate diagnosis kit (Roche, Basel, Switzerland). The culture supernatants were directly applied to the quantification.

PGE_2 was measured by Prostaglandin E_2 EIA Kit Monoclonal (Oxford Biochemical Research, Oxford, MI, USA). Culture supernatants were used for measurements.

Statistics

All statistical tests were performed using R software (version 3.0.0; R Foundation for Statistical Computing, Vienna, Austria [http://www.R-project.org/]). A non-paired two-tailed Student's t-test was used to compare the *in vitro P. falciparum* studies.

Results

Exogenous addition of SA

Exogenous addition of SA did not alter parasite growth (Fig. 9), similar to the result of *T. gondii* in chapter 3. This is probably because the parasite already contained enough levels of SA, or SA does not affect the parasite growth.

Establishment and analysis of SA deficient parasites

Because the addition of SA did not have any significant phenotype, we transfected *P. falciparum* with the *nahG* gene, that encodes an SA-degrading enzyme found in a plant pathogenic bacterium, *Pseudomonas putida* (Gaffney *et al.* 1993). The

expression of this gene product was confirmed by Western blotting using 2× myc-tag at the carboxyl-terminus of nahG (Fig. 10). The SA concentration of *nahG*-transfectants was decreased compared with *gfp*-transfected controls, though we could find no statistical difference (Fig. 11).

The growth kinetics and cell cycle of the SA-deficient parasites

The growth kinetics and cell cycle progression of the *nahG*-transfectant was not different from *gfp*-transfected control (Fig. 12, 13). Taken together the result of addition of SA to the parasite culture (Fig. 9), these results indicated that the concentration of SA had no effect on the parasite growth and cell cycle development.

Prostaglandin concentrations of SA-deficient parasites

SA is a member of NSAIDs and modulates PGE_2 levels of animals. Furthermore, *Plasmodium* produces its own PGs, of which PGE_2 is predicted to interfere with the immune system of a host (Kubata *et al.* 1998). We hypothesized that malaria parasites could modulate the concentration of PGE_2 in hosts by SA and its own production of PGE_2. Therefore, we next quantified the concentration of parasite prostaglandins. Surprisingly, the PGE_2 concentration decreased significantly when the *nahG* gene was transfected into parasites (Fig. 14).

Discussion

SA manipulation of *P. falciparum* showed no effect on growth. It was consistent with the result of *T. gondii* indicated in chapter 3. As mentioned above, SA accumulation is much higher than other plant hormones. This explains why we could

not detect the effects on growth by the exogenous addition of SA. In the case of land plants, such effect usually only occurs when the compound is added at excessive concentrations many times higher than the endogenous concentration. For example, the 90% inhibitory concentration (IC_{90}) of ABA to *O. sativa* growth was 10 μM, which was almost 300-fold higher than the native production of 9.2 ng/g fresh weight, approximately 0.03 μM (Hoffmann-Benning & Kende 1992). Nevertheless, SA deficiency by *nahG*-introduction also showed no effect on parasite proliferation. Considering all, SA has neither positive nor negative effects on parasites growth, rather than that the manipulation of SA concentration was not enough.

Although SA has been studied for 100 years, intense studies focused only on the anti-inflammatory effects on animals. In contrast, there have been few studies of signaling pathways and biosynthesis compared with other plant hormones (Kelley & Estelle 2012). BA and ICS pathways were identified as the SA synthetic routes in land plants, but the enzymes in the BA and ICS pathways have not been identified in any of apicomplexan genomes. Additionally, although NPR3 and NPR4 were identified as the SA receptors recently (Fu *et al.* 2012), I also could not identify the orthologues of NPR1, 3, or 4 in any apicomplexan genome database. Because of the large phylogenetic distance between land plants and Apicomplexa, apicomplexans possibly lost the homologs of NPR3 and NPR4, or the land plants have developed those genes after they diverged from the common ancestor of Apicomplexa and land plants. Similar discussion was previously suggested in apicomplexan ABA synthesis pathway (Nagamune *et al.* 2008).

Because we did not detect any synthetic enzymes or receptors for SA in *Plasmodium* spp. genomes, we transfected *P. falciparum* with the SA degrading gene,

nahG, originally identified as a pathogenic factor of plant-infecting bacterium *Pseudomonas putida*, which encodes SA hydroxylase, that catalyzes SA to biologically inactive catechol (Gaffney *et al.* 1993). The *nahG*-transfectant decreased SA concentration by approximately 50%, which was relatively ineffective compared with the results of the plant transformants (Gaffney *et al.* 1993). This might reflect differences in the intracellular environment (e.g., ions and pH) between land plants and *Plasmodium* spp. However, the mutants of *P. falciparum* significantly decreased the endogenous production of PGE_2, suggesting that the deficiency was sufficient to explain the role of SA in *Plasmodium* spp. as a signal molecule.

PGE_2 usually functions as an active mediator of inflammation in mammals. The most remarkable effect of this molecule is inductions of fever and pain. PGE_2 is greatly induced at the site of inflammation, leads pain, and if delivered to the central nerve system, it induces fever onset through the PGE_2 receptor 3 expressed by neurons in the median preoptic nucleus (Lazarus *et al*. 2007). PGE_2 also interferes innate acquired immunity. Considering the effect of SA and PGE_2, I next analyzed the *in vivo* function of SA and PGE_2 by murine malaria parasite *P. berghei*.

5. SALICYLIC ACID AFFECTS THE CEREBRAL MALARIA OUTCOME

Introduction

Within 5 *Plasmodium* species infectious to human, especially infection of *P. falciparum* causes the malignant complicated malaria which develops cerebrovascular disorder as the main mark. The severe malaria with cerebral disorder caused by the sequestration of infected erythrocytes leads the clinical symptoms such as abnormal behavior, impairment of consciousness, seizures and coma followed by rapid death, called cerebral malaria (CM, Idro *et al*. 2010). Addition to the importance, *P. falciparum* is only one malaria parasite which can be cultured *in vitro* continuously. By these reasons, extensive studies including mechanisms of invasion to the host cell, metabolisms and membrane trafficking, cellular division to the sexual and asexual form, protein transport to outside/inside of the parasite, and ultrafine structure have conducted on *P. falciparum*. However, the immunity and pathogenicity of the cerebral malaria were hard to study because of lacking *in vivo* model of *P. falciparum*.

P. berghei is a rodent malaria parasite and used worldwide as a model of human malaria parasite (Ramos-Summerford *et al.* 2014). Especially, *P. berghei* strain ANKA infection on mouse C57BL/6 strain leads rapid cerebral disorder, and mouse died within a week even the parasitemia is not extremely high (Promeneur *et al.* 2013). The mouse shows significant neurologic symptom on behavior as the case of *P. falciparum* infection. Therefore, this model is widely accepted as a model of human cerebral malaria, namely experimental cerebral malaria (ECM).

CM is caused by the sequestration of blood cells or disruption of the blood brain barrier (BBB) on small vessels of the brain. Furthermore, the aggravating role of

proinflammatory cytokines, such as Interferon (IFN) -γ, Tumor necrosis factor (TNF) -α, and Interleukin (IL) -12, to CM development is also widely accepted (Hunt & Grau 2003; Idro *et al*. 2010). The mechanisms of CM development have been investigated by clinical observation of human patients as well as laboratory animal models with *P. berghei* strain ANKA. For example, the elevation of serum levels of IFN-γ was associated with the severity of acute malaria in Asian and African patients (Ho *et al*. 1995; Ringwald *et al*. 1991), and both *in vivo* IFN-γ neutralization and knock-out of IFN-γ receptor enhanced resistance against ECM (Amani *et al*. 2000; Yañez *et al*. 1996). TNF-α also has a crucial role in the pathogenesis of CM as administration of anti-TNF-α antibody completely protected against *P. berghei* strain ANKA infection (Grau *et al.* 1987). In addition to these immune responses, *Plasmodium* actively modulates the immune system of its host. Hemozoin (malarial pigment) effectively suppresses the functions of dendritic cells (Keller *et al.* 2004). PGE_2 might also suppress the host immunity by switching cytokines to a humoral immunity dominant immune status (Kalinski 2012). However, larges concerning the immune interference of malaria parasite are still unclear.

PGE_2 is also involved in the development and aggregation of CM. In a field study, an inverse relationship between the concentration of plasma PGE_2 and the severity of disease by *P. falciparum* infection was reported in Gabonese children (Perkins *et al*. 2001). In mouse model, the protective role of PGE_2 was also proven by administration of NSAIDs to infected mice, where the subscription of NSAIDs aggravates the ECM and mortality (Ball *et al*. 2004; Xiao *et al*. 1999). These findings indicate the importance of PGE_2 in the development of CM, and this effect can be explained by the suppressive activity of PGE_2 on the production of proinflammatory

cytokines (Kalinski 2012).

The study of *P. falciparum* showed that the PGE_2 concentration of parasite own was regulated by the level of SA (chapter 4). Together with the fact that PGE_2 is known to regulate the outcome of CM and ECM, the effect of parasite SA to the CM outcome is strongly suggested. In this chapter, therefore, I established the *nahG* expressing *P. berghei* ANKA and examined the ECM outcome.

Materials & Methods

Parasites and animals

Experiments including mouse survival test, ECM assessment, cytokines, and prostaglandin quantification were performed using the C57BL/6 mouse strain. Female mice, 6–9 weeks old, were purchased from Japan SLC and used for experiments.

Establishment of salicylic acid-deficient parasites

The *nahG* expressing vector was constructed with the pL0006 vector (distributed from MR4), and *nahG* was also driven by pCRT and PbDT as described in chapter 4. The vector was linearized and recombinated into the *230p* gene locus, which has no known function (Lin *et al*. 2011). The *P. berghei* ANKA transfectants were cloned by limiting dilution as described previously (Janse *et al*. 2006). The control parasites transfected with *gfp* were made with the same way. The expression of *nahG* was confirmed by Western blotting with rabbit anti-cMyc antibody and anti-HSP-90 antibody as control.

Mice mortality assay

C57BL/6 mice received 10^4 parasitized red blood cells intravenously. Mouse survival, weight, parasitemia, and clinical signs were checked every day. After mice died, brain hemorrhage was observed to diagnose the cause of death.

Evaluation of ECM

To evaluate the disruption of BBB, a dye leakage test was performed as previously described (Penet *et al*. 2008). Briefly, mice 6 days post-infection were intravenously injected with 100 µl of Evans blue dye (1% w/v in PBS; Sigma Aldrich). After 1 h, mice were anesthetized and euthanized to collect whole brains. The brains were photographed, weighed, and transferred into 2 ml formamide (Wako Chemicals, Osaka, Japan). Samples were incubated for 48 h at 37 °C and dye extravasation was determined by measurement of optical density (OD) at 640 nm. The values were normalized by tissue weight.

To evaluate the sequestration of vessels in brains, the samples were observed histologically. Mice 6 days post-infection were anesthetized, perfused through the heart with 5 ml of PBS followed by 5 ml of ice-cold phosphate-buffered 4% paraformaldehyde solution (PFA; Wako Chemicals) for fixation. Brains were transferred into 4% PFA and fixed overnight at 4 °C. Brain slices stained with hematoxylin and eosin were observed by microscopy. To quantify the sequestration of vessels, mice cerebellums were photographed at low magnification. All vessels and sequestrated vessels were counted, and the sequestration ratio was calculated.

Prostaglandin and cytokine quantification

PGE_2 was measured by Prostaglandin E_2 EIA Kit Monoclonal (Oxford Biochemical Research, Oxford, MI, USA). Plasma from heparin-treated whole blood was used for measurements. Mouse plasma cytokines from the animals 6 days post-infection were collected as above, analyzed by the Bio-Plex Pro Mouse Cytokine 23-plex Assay and Th1/Th2 Assay Kit (Bio-Rad, Hercules, CA, USA).

Statistics

All statistical tests were performed using R software (version 3.0.0; R Foundation for Statistical Computing, Vienna, Austria [http://www.R-project.org/]). The Mann–Whitney U-test was used for non-parametric comparison for Evans-blue leakage test, plasma prostaglandins, and cytokine quantification and clinical signs (weight, parasitemia, and hematocrit). Bonferroni correction was used for multiple comparisons. The log-rank test was used to compare the survival curves of infected mice. Kaplan–Meier survival curves of parasite-challenged mice and box graphs of *in vivo* examinations were also depicted by R software.

Results

Influence of SA deficiency on mouse survival

To investigate the effect of SA *in vivo*, we also established SA deficient *P. berghei* ANKA by transfection with *nahG*. First, we observed the pathogenic difference of the SA deficiency. C57BL/6 mice were intravenously challenged with 10^4 parasitized RBCs. As indicated in Fig. 15, mice challenged with the *nahG*-parasites had significantly increased the mortality compared with the *gfp*-parasite group ($p < 0.05$,

Log-lank test). We used three independent *nahG*-transfected clones and confirmed similar results (data not shown). Both *nahG* and *gfp* parasites induced ECM, resulting in a neurological syndrome characterized by clinical signs including ataxia, convulsions, and coma, followed by death. However, the onset of ECM was shortened by 1 or 2 days by infection with *nahG*-transfectant compared with the control parasites.

Influence of *in vivo* SA deficiency to ECM

Since there seemed to be a difference between the *nahG* and *gfp*-parasites infection in the severity of ECM, we histologically analyzed the brains of infected mice to evaluate ECM including the sequestration of micro vessels and hemorrhage (Fig. 16). The cerebellum of each mouse was stained with hematoxylin and eosin, and observed for pathology. The cerebellum of mice infected with *nahG*-transfectants showed obvious leucocyte sequestration in small vessels (Fig. 16A). This pathological observation was reported as a typical symptom of ECM (White *et al*. 2010). Mice infected with *gfp*-transfectants showed slight hemorrhage but no sequestration of leukocytes, suggesting an under-developed ECM (Fig. 16B). We did not observe pathological changes including hemorrhage and sequestration of leucocytes in vessels in uninfected control mice (Fig. 16C). I quantified the sequestration ratio of vessels. I observed at least 500 vessels per mouse, and found that the mice infected with *nahG*-transfectants showed significant sequestration (Fig. 17).

Next, we quantified the severity of ECM by Evans blue leakage assay (Fig. 18). Evans-blue leakage was significantly distinct in infected mice ($p < 0.01$, *nahG* vs. control and GFP vs. control; $p < 0.05$, *nahG* vs. GFP) indicating that *nahG*-transfectants severely disrupted the BBB in ECM. Mouse parasitemia and body weight kinetics were

also measured (Fig. 19 and 20). Parasitemia at day 6 after infection when mice infected with *nahG*-transfectant but not *gfp* showed ECM signs, was not significantly different each other, suggesting that differences in the growth capacity of parasites did not explain differences in clinical changes (Fig. 19). Hematocrit scores at the same day were also equivalent (Fig. 21). Mouse weight did not show differences, or even shifted higher in *nahG*-transfectants infection (Fig. 20). Considering all, the differences in mortality may be explained by the occurrence of ECM by infection with *nahG*-transfectants.

Quantification of prostaglandins and cytokines in infected mouse plasma

The ECM evaluation showed increasing the severity in *nahG*-transfected parasite, and the *in vitro* results by *P. falciparum* indicated that a deficiency in the production of SA affected the production of PGE_2 by the parasite. So, we decided to investigate the concentrations of plasma PGE_2 from mice and several cytokines influenced by PGE_2. Whole blood from mice (6 days post-infection) was collected for quantification of PGE_2 and cytokines. The day mice showed clinical signs, but no differences in parasitemia or hematocrit were detected as shown above (Fig. 19,21). The plasma PGE_2 concentration of mice infected with *nahG*-transfected parasites were decreased compared with the *gfp*-transfectant or uninfected control, though there were no significance ($p < 0.05$, Fig. 22A). This shortage in PGE_2 was consistent with the *in vitro* results.

The concentration of proinflammatory (T helper-1, Th1) cytokines in *nahG*-transfectant-infected mice changed dramatically. IFN-γ and IL-2 increased significantly ($p < 0.01$, Fig. 22B and D, respectively). IL-1β levels were also increased

by infection of *nahG*-parasites (Fig. 22E, $p < 0.05$). Other major proinflammatory cytokines, TNF-α and IL-12, showed similar shifts although they were not statistically significant (Figs. 22C and F, respectively). Although the concentration of Th2 cytokine, IL-10, did not change significantly (Fig. 23A), IL-4 and IL-5 were significantly elevated in mice infected with *nahG*-parasites (Figs. 23B and C, respectively). An inflammatory chemokine Monocyte chemoatract protein (MCP)-1 was significantly decreased in *nahG*-transfectant infection ($p < 0.05$, Fig. 23D).

Discussion

PGE_2 usually functions as an active mediator of inflammation in mammals. It is released from the whole cells, mainly vascular endothelial cells, fibroblasts, and mast cells. PGE_2 has complex activities in inflammation. It induces fever and pain, and has a suppressive activity on both innate and acquired immune reactions. During innate immunity, PGE_2 suppresses the functions and differentiation of natural killer cells, macrophages, granulocytes, and mast cells (Kalinski 2012). For example, it was reported that the cytolytic activity of natural killer cells was suppressed by PGE_2 by a mechanism involving the suppression of IL-2, IL-12, and IL-15 (Walker & Rotondo 2004; Joshi *et al*. 2001). Furthermore, macrophage functions are directly suppressed by PGE_2 receptor 2-dependent signaling (Aronoff et al. 2004). In acquired immunity, PGE_2 inhibits the production of IL-2 by T cells and IL-2 responsiveness (Kalinski 2012). It also suppresses T-cell activation and proliferation, and shifts the pattern of $CD4^+$ T-cell responses from Th1 to Th2 and Th17 cells (Kalinski 2012). PGE_2 directly prevents $CD4^+$ T-cell production of IFN-γ but not Th2 cytokines such as IL-4 and IL-5 in mice

and humans (Betz & Fox 1991; Snijdewint *et al.* 1993). Additionally, it prevents differentiation of $CD4^+$ T-cell to Th1 by suppressing monocyte and dendritic cell production of IL-12 (Kalinski 2012; van der Pouw Kraan *et al*. 1995).

To investigate the function of parasite SA and induced PGE_2, we established *nahG*-expressing *P. berghei* ANKA, and found that parasite virulence was enhanced significantly. The *nahG*-expressing *P. berghei* killed mice in a shorter period than the control parasite which express *gfp*. The cause of death was diagnosed as ECM by the presence of neurological syndromes, clinical behaviors, histological observation, and dye leakage test. Especially the dye leakage test provided the statistical evidence that the severity of ECM was significantly different depending on the carrying genes (*nahG* or *gfp*) of infecting parasites. Also the histological observation indicated the aggregating sequestration of blood cells to the microvessel of the brain in *nahG*-expressing *P. berghei* infection.

The early onset of ECM suggested that SA produced by the parasite affected the *in vivo* virulence of *Plasmodium*. The mechanism might be PGE_2 dependent, because PGE_2 protects against CM, and PGE_2 significantly elevated at least the culture model of *P. falciparum* whereas any other differences, for example, parasitemia, body weight, and hematocrit, were not observed. The importance of PGE_2 to the protection against CM onset was verified by both field and laboratory studies (Perkins *et al*. 2001; Ball *et al*. 2004; Xiao *et al*. 1999). These findings indicate the importance of PGE_2 in the development of CM, and this effect can be explained by the suppressive activity of PGE_2 on the production of proinflammatory cytokines (Kalinski 2012). Our study demonstrated that infection with the *nahG*-transfectant, (SA-deficient parasite) induced the up-regulation of proinflammatory cytokines including IFN-γ, IL-1β, IL-2, and IL-12,

while the parasitemia of the same time point were almost uniform (Fig. 19). It probably suggests that the SA-deficiency released the lock on the PGE_2-dependent immune suppression, because the SA-deficiency in *P. falciparum* strongly connected to the PGE_2 decrease. Another proinflammatory chemokine, MCP-1, showed an uncoordinated decrease, different from other proinflammatory cytokines. Generally, at the site of infection, MCP-1 cooperatively elevated with other inflammatory substances and involve in the inflammation establishment. In addition, a previous study demonstrated that the release of MCP-1 from mast cells was promoted by PGE_2 stimulation (Nakayama *et al*. 2006), and that mast cells were thought to be the dominant source of MCP-1 (Carollo *et al*. 2001). Thus the MCP-1 opposite decrease in *nahG* transfected parasite infection suggests that fewer PGE_2 production of the parasite possibly allow other cytokines increase, whereas it might reduce MCP-1 induction. Enhanced IL-4 and 5 production in mouse plasma from *nahG*-transfectant-infected mice is controversial because Th2 cytokines are generally protective against CM development, especially IL-4. However, a previous study reported that IL-4 was not associated with ECM pathogenesis (Yañez *et al.* 1996). Furthermore, few studies have focused on the role of IL-5 in the complexity of severe malaria including CM. IL-5 production was not different between ECM-susceptible and -resistant mouse strains (de Kossodo & Grau 1993). The present study observed the significant induction of IL-4 and IL-5 and might reflect unknown cytokine functions in ECM development, which might be highlighted by the PGE_2-deficient conditions. Further analyses are needed to understand the role of these cytokines for the pathogenesis of CM.

The plasma PGE_2 levels were not significant between the mutants of *P. berghei* ANKA *in vivo*. It suggests the difference of PGE_2 is present only at the

microenvironment of infection. To investigate this possibility, the analysis using PGE_2-receptor knock out mouse will be needed in the future study.

In this chapter, I observed the clinical outcome of ECM was different between SA-deficient and mock (*gfp*-expressing) parasites. These data highlighted the highly constructed system of parasite to modify and control the host immunity and disease development.

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TABLE

FIGURES

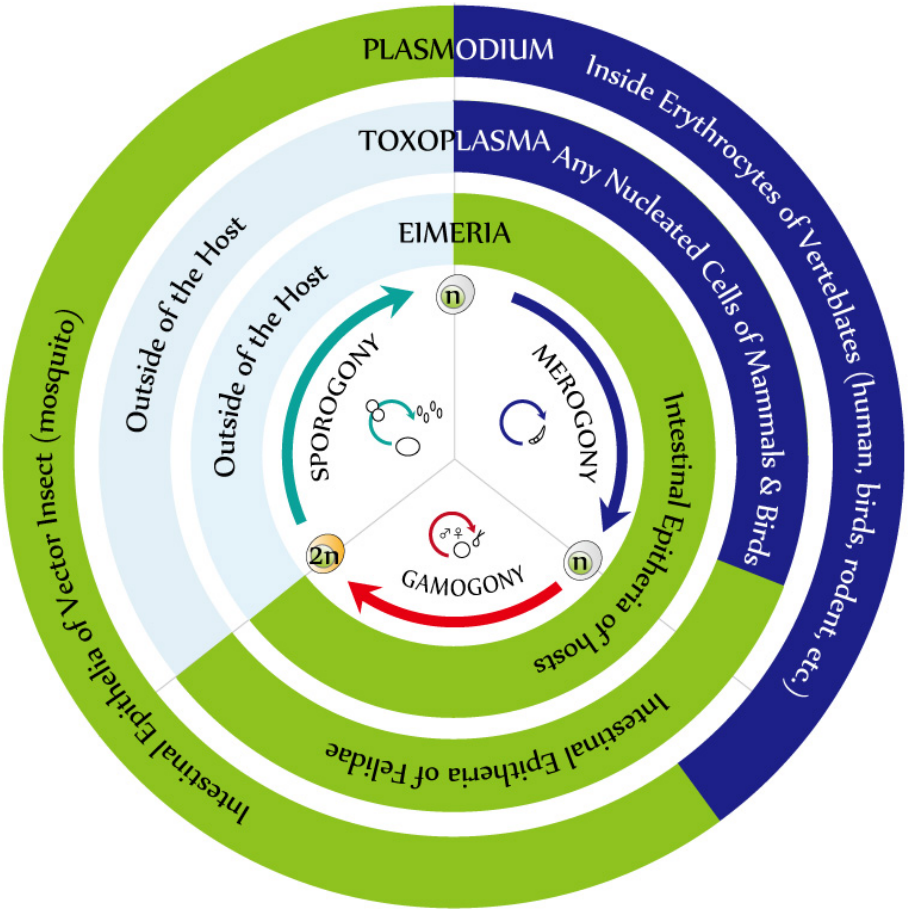

Fig. 1. The lifecycle of Apicomplexa.
The lifecycles of *Plasmodium* spp., *Eimeria* spp. and *T. gondii* are shown. The life cycles of this phylum basically consists of 3 stages: merogony, gamogony and sporogony. The lifecycles of *Plasmodium* spp., *Eimeria* spp. and *T. gondii* are plotted onto the schematic apicomplexan lifecycle.

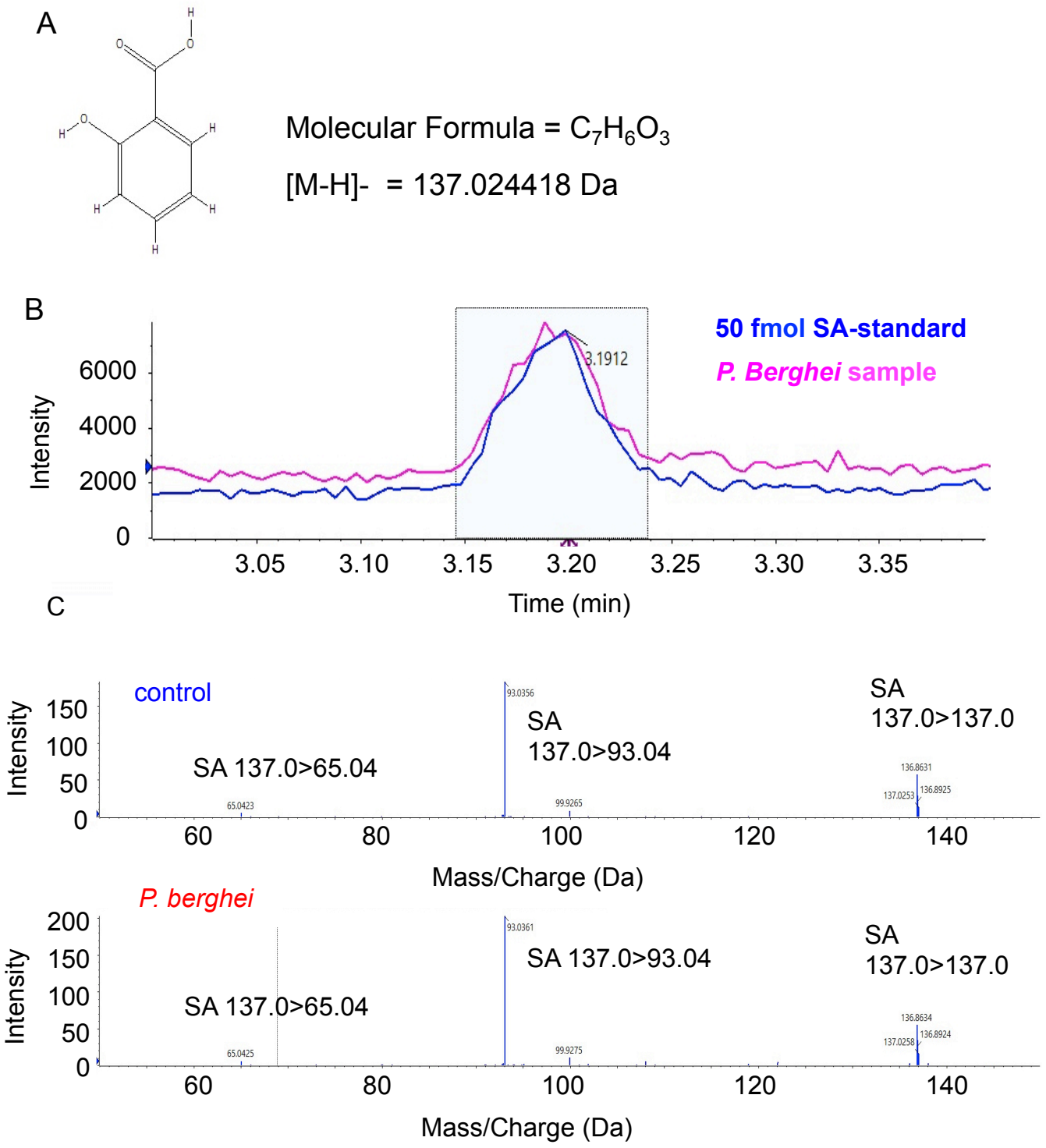

Fig. 5. Identification of salicylic acid with UHPLC/HPLC/Triple TOF mass spectrometry system.

Plasmodium berghei strain ANKA was purified from infected mice blood, salicylic acid was extracted, and analyzed by LC-triple TOF mass spectrometry. (A) Structural formula of SA. (B) LC chromatogram of SA standard (control) and *P. berghei* ANKA sample. (C) Fragmentation analysis of peaks in (B) (colored in aqua). Collision energy was 20 eV.

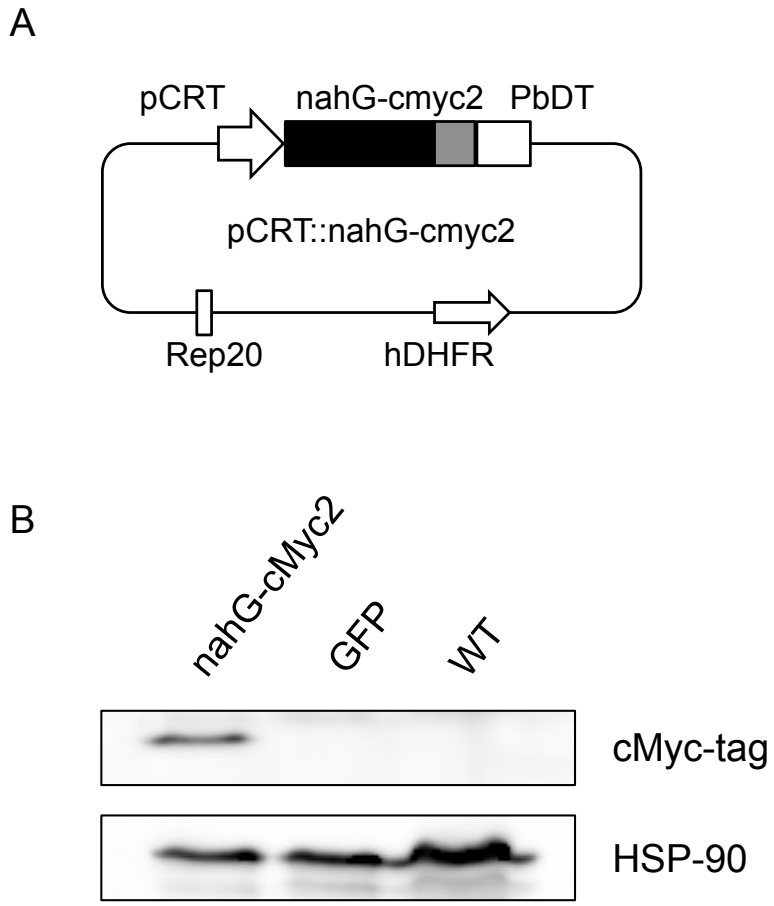

Fig. 10. Western blotting of the *nahG* product.
(A) Construction of expression vector of *nahG* in *P. falciparum*. *P. falciparum* was transfected with the SA degrading gene, *nahG,* driven by *P. falciparum* chloroquine resistant transporter gene 5' UTR (pCRT) and *P. berghei* dihydrofolate reductase gene 3'UTR (PbDT), fused into attR4-attR3 site of the destination vector pCHD43 (II), and transfected by electroporation. The control parasite line transfected with *gfp* was also made with same way. **(B)** Western blotting of the transfectants. The expression of *nahG* was confirmed by Western blotting with rabbit anti-cMyc antibody, and anti-HSP-90 antibody as internal control.

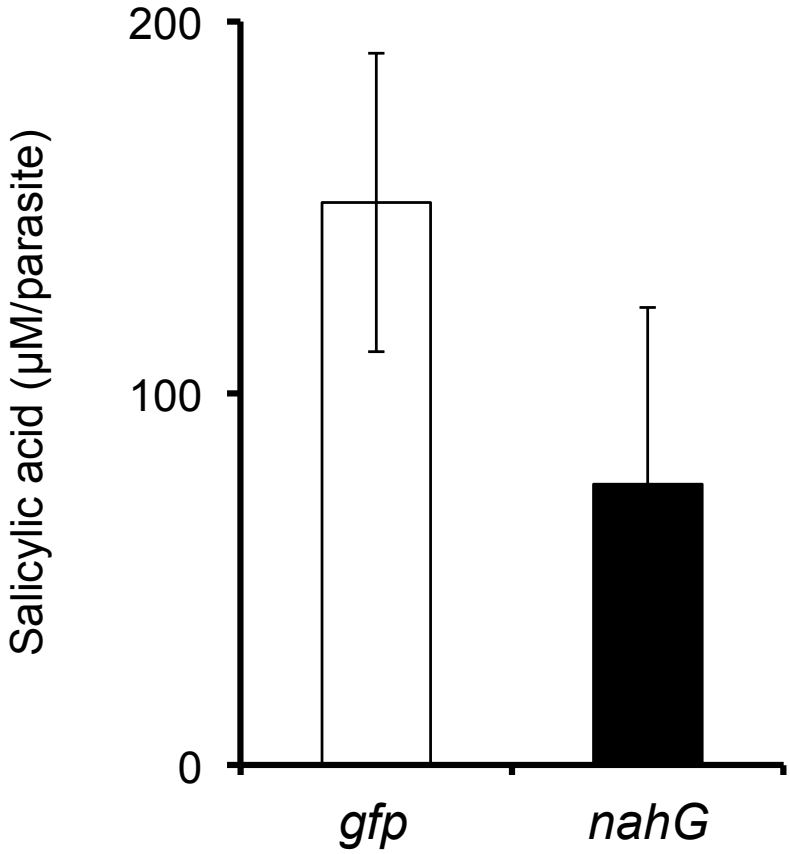

Fig. 11. The SA concentration of *nahG*-transfectants.
The SA concentration of *nahG*-transfectant was compared to that of *gfp*-transfected control. The culture supernatants of parasites at similar parasitemia were examined, and the data was normalized by parasitemia. The SA concentration of *nahG*-transfectant was decreased compared with *gfp*-transfected controls, though we could not find statistical difference. Bar indicates SD. n=3

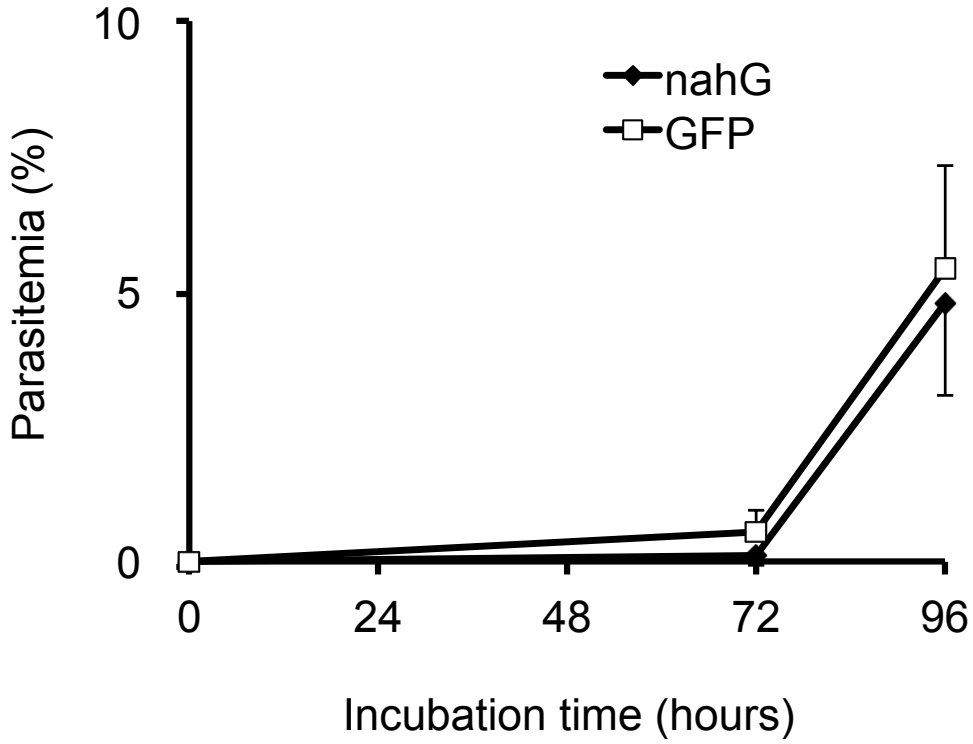

Fig. 12. The growth kinetics of the *nahG*-transfectant.
The *nahG* and *gfp*-transfected parasites were cultured, and synchronized by 5% D-sorbitol twice. Then the parasites were adjusted to 0.1% of parasitemia, and growth were calculated by counting parasites on thin blood smear. The transfection with *nahG* to *P. falciparum* did not show effect on growth *in vitro,* comparing to the *gfp*-transfectant control. Bar indicates SD. n=3

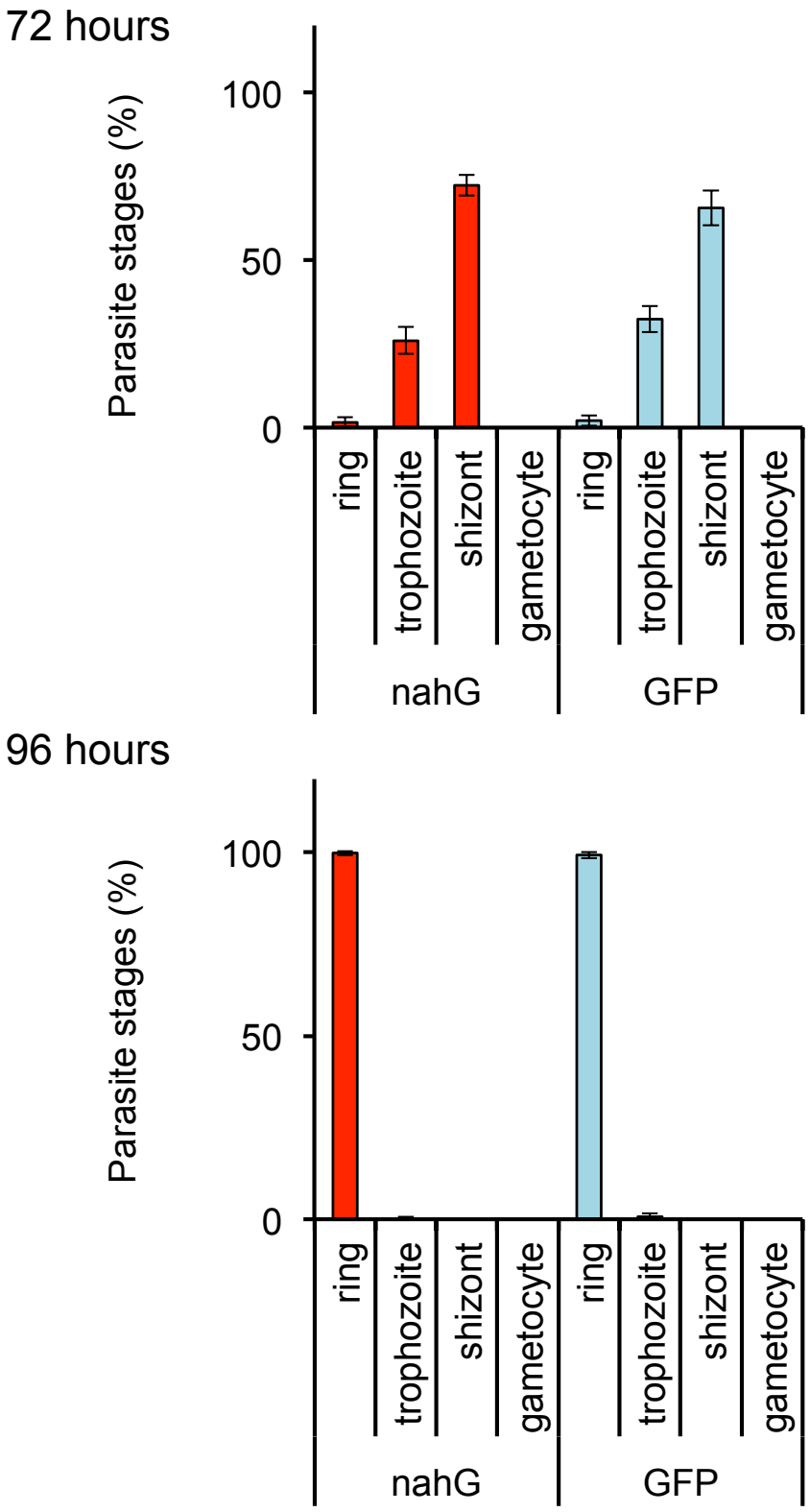

Fig. 13. The cell cycle progression of the *nahG*-transfectant.
The *nahG* and *gfp*-transfected parasites were cultured, and synchronized by 5% D-sorbitol twice. Then the parasites were adjusted to 0.1% of parasitemia, and thin blood smear was made to observe the parasite stage. The transfection with *nahG* to *P. falciparum* did not show effect on cell cycle *in vitro,* comparing to the *gfp*-transfectant control. Bar indicates SD. n=3

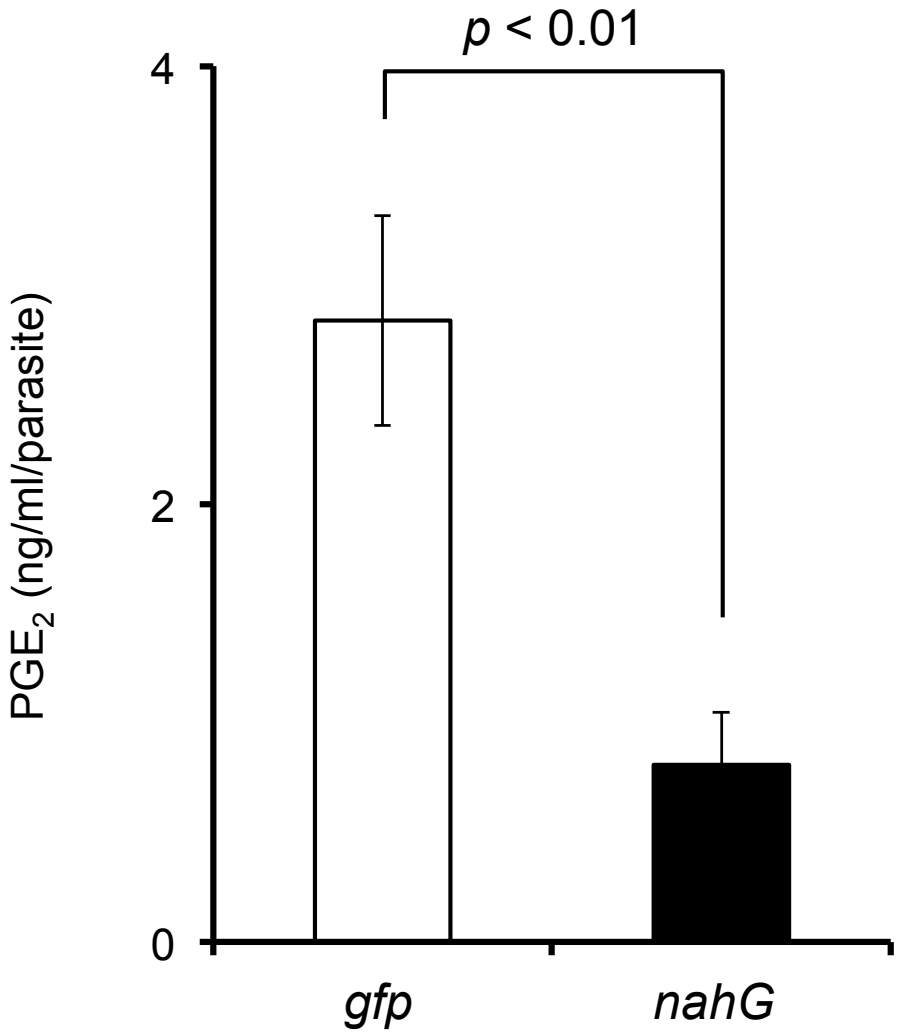

Fig. 14. Prostaglandin concentrations of SA-deficient parasites.
The *nahG* and *gfp*-transfectants were cultured *in vitro*, and the extracellular Prostaglandin E_2 (PGE_2) was quantified from the culture supernatant. The value was normalized by parasitemia. The PGE_2 concentration of *nahG*-transfectant decreased significantly than *gfp*-transfectant. The significance was tested by two-tailed Student's t-test. Bar: SD. n=3.

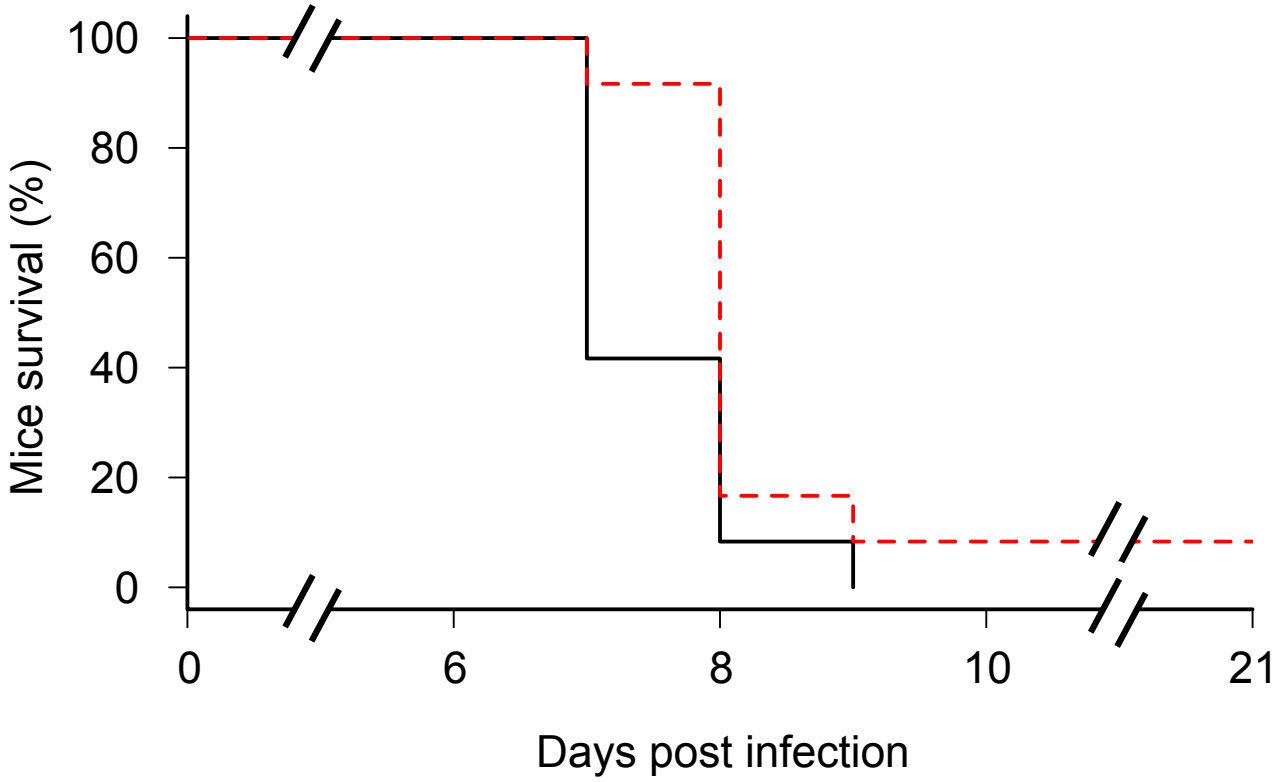

Fig. 15. Influence of SA deficiency on mouse survival
SA deficient *P. berghei* ANKA was established by transfection with *nahG*. C57BL/6 mice were intravenously challenged with 10^4 parasitized RBCs. Mice challenged with *nahG*-parasites significantly shortened the survival period compared with the *gfp*-parasite group ($p<0.05$, Log-lank test). We used three independent *nahG*-transfected clones and confirmed similar results (data not shown).

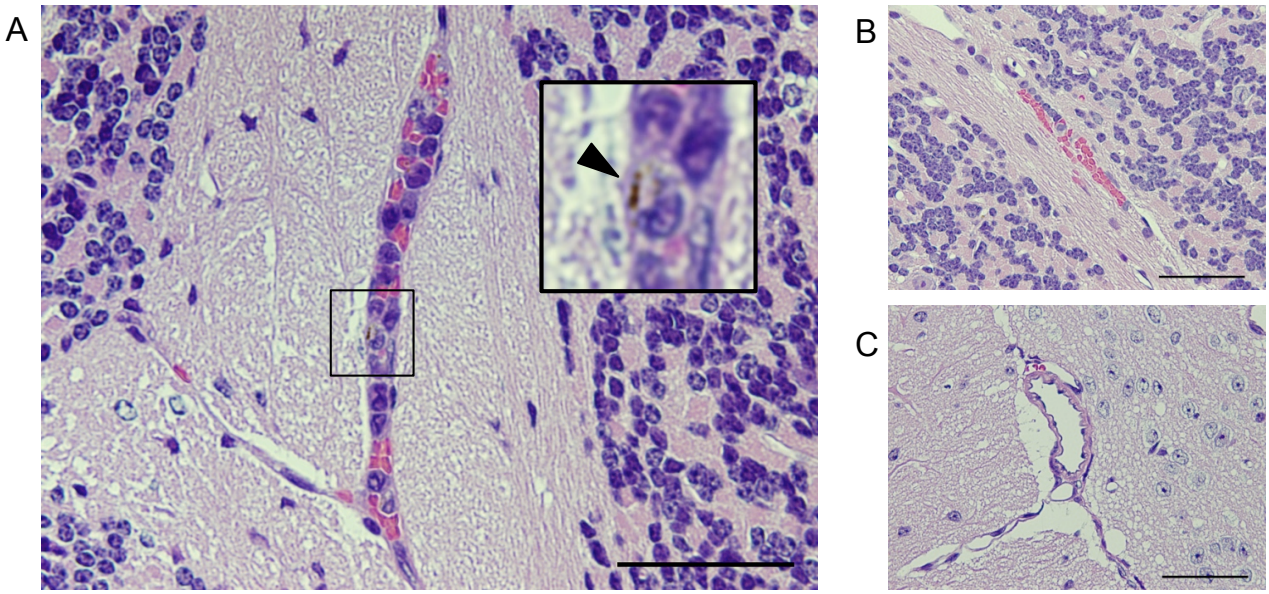

Fig. 16. Influence of *in vivo* SA deficiency to experimental cerebral malaria
The cerebellum of mice challenged by *nahG* or *gfp*-transfected parasites, or naïve mice (control) was histologically examined. The cerebellum of each mouse was stained with hematoxylin and eosin and observed for pathology. **(A)** The cerebellum of mice infected with *nahG*-transfectants showed significant leucocyte sequestration in small vessels. This pathological observation was similar to a typical symptom of experimental cerebral malaria (ECM). Inset image shows a higher magnification of the boxed portion. Phagocytized hemozoin is observed (arrowhead). **(B)** Mice infected with *gfp*-transfectants showed slight hemorrhage but no sequestration of leukocytes, suggesting an under-developed ECM. **(C)** Any pathological changes, including hemorrhage and sequestration of leucocytes in vessels, was not observed in uninfected control mice. C57BL/6 mice, female, 6 days after infection were used. Bar: 50 μm.

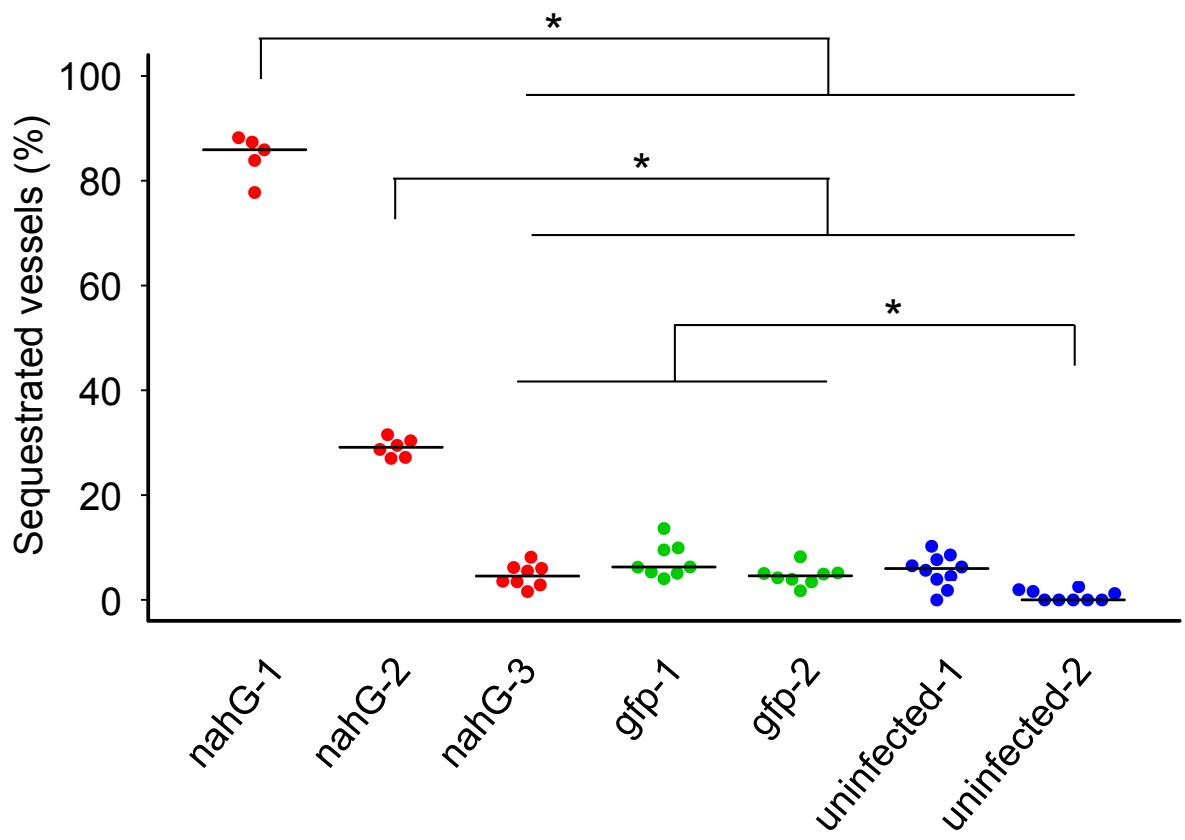

Fig. 17. Quantification of sequestrated brain vessels
Brains of mice infected by *nahG* or *gfp*-transfected parasites, or uninfected (control) mice were perfused with PBS and fixed with 4% PFA. Sliced sections were stained by hematoxylin and eosin. Cerebellums of the brains were photographed, and 5 to 10 pictures per mice were counted (n>100 for each picture). A Mann-Whitney U-test with the Bonferroni's correction was used, and significant (p<0.05) differences were denoted by asterisks.

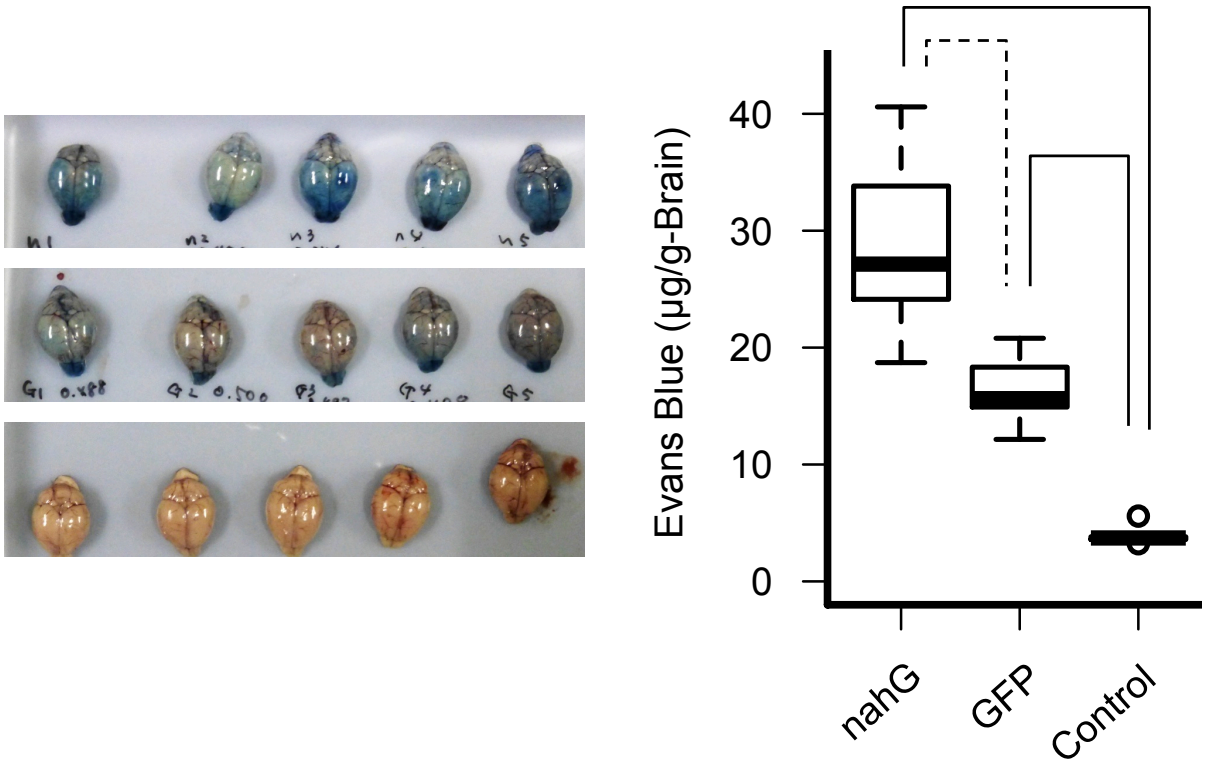

Fig. 18. Influence of *in vivo* SA deficiency to ECM
Evans blue leakage analysis of the severity of cerebral malaria. Brains from mice infected with *nahG*- (left-upper), *gfp*- (left-middle) expressing parasites and uninfected controls (left-bottom) were photographed. Dye leakage was quantified (right). Mice (n=5) were sacrificed at 6 days post-infection. Solid-line, $p<0.01$; dashed-line, $p<0.05$. C57BL/6 mice at six days post-infection were used for all experiments. Mann Whitney U test was used for statistics.

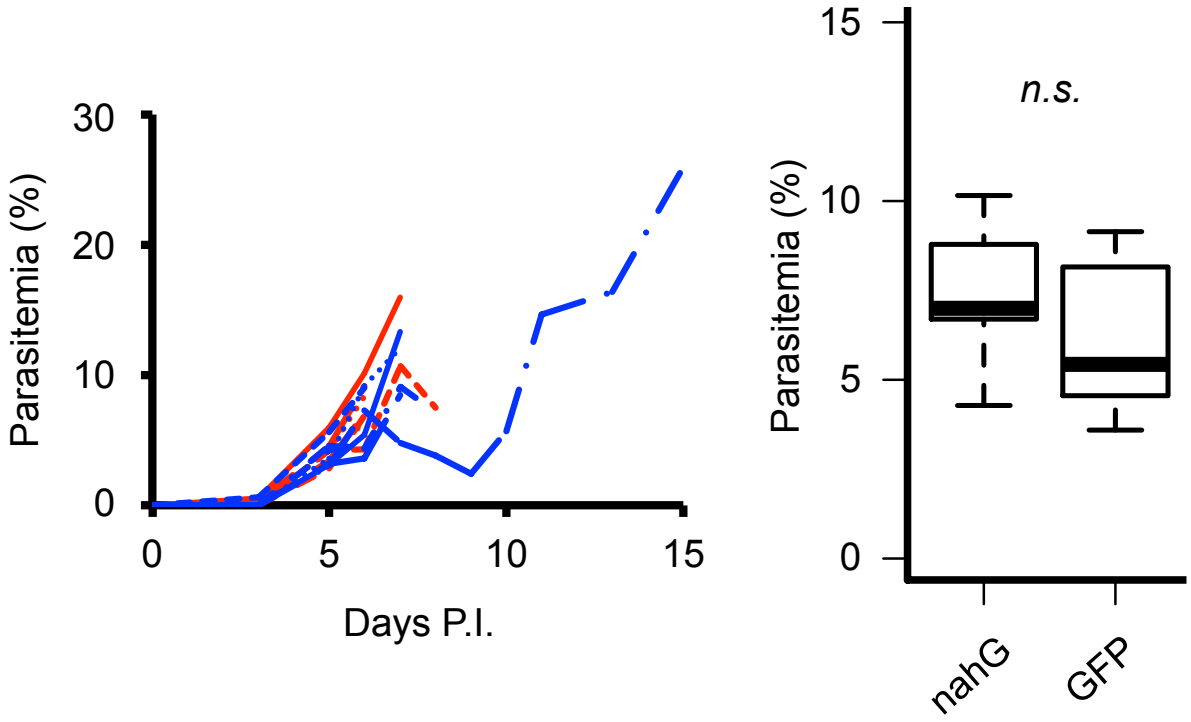

Fig. 19. Mouse parasitemia
Each graph indicates the individual mouse infected by *nahG* (red) or *gfp* (blue)-expressing parasites (left). Parasitemia of *nahG* or *gfp*-expressing parasites at day 6 post infection, when the clinical signs were most significant (right). The plasma PGE_2 and cytokines were quantified at this time point. C57BL/6 mice were used for experiments. Mann Whitney U test was used for statistics.

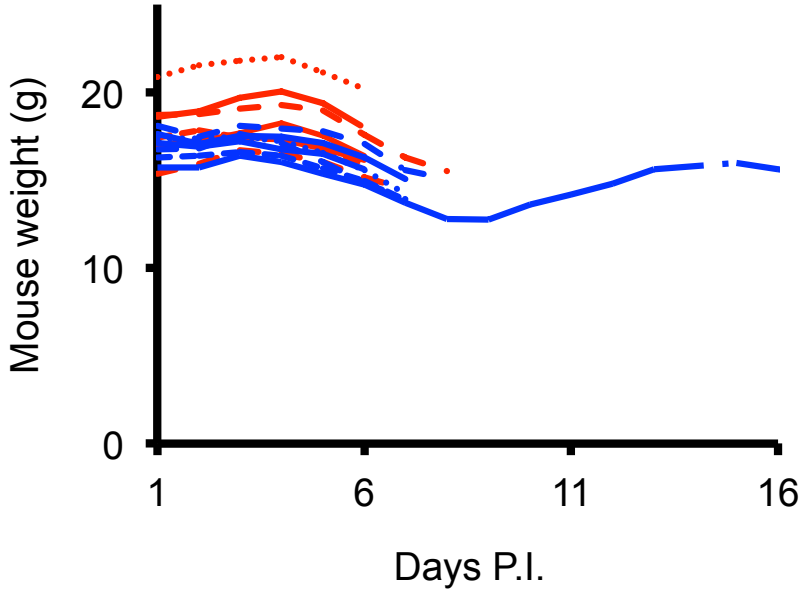

Fig. 20. Mouse body weight kinetics
Animal weight gain/loss are shown. Each graph indicates the individual mouse infected by *nahG* (red) or *gfp* (blue)-expressing parasites. C57BL/6 mice were used for experiments.

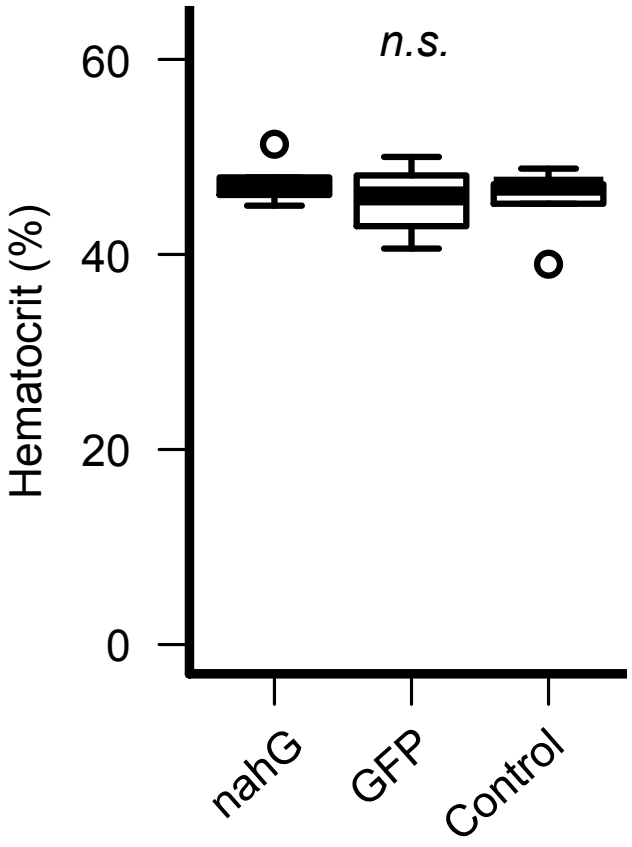

Fig. 21. Hematocrit scores of infected mice.
Hematocrit scores of infected mice at day 6 post infection, when the clinical signs were most significant, are shown. The hematocrit scores show no significant difference. C57BL/6 mice were used for experiments. Mann Whitney U test was used for statistics.

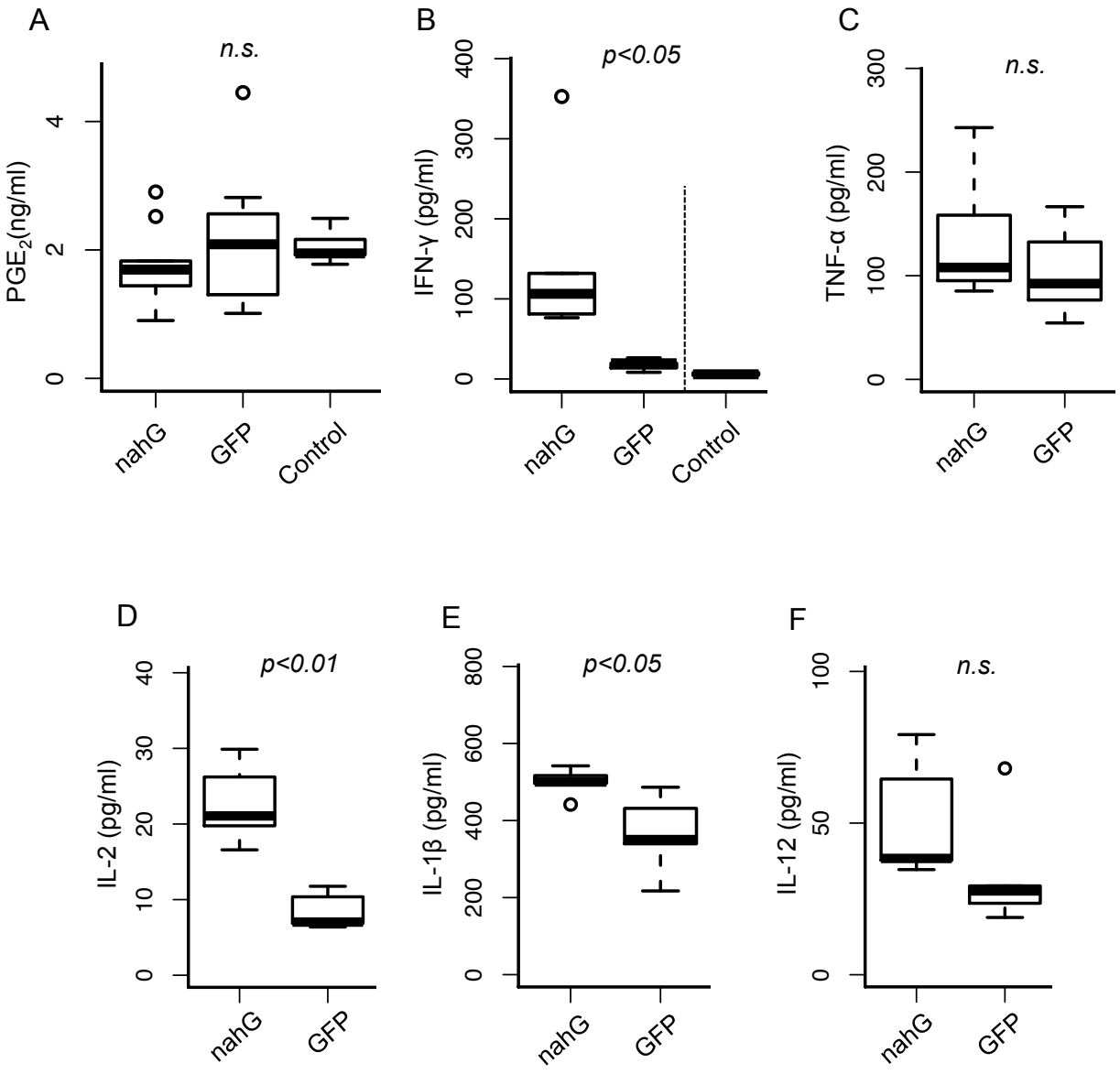

Fig. 22. Plasma proinflammatory cytokines and prostaglandin E_2 (PGE_2) from infected mice.

Plasma proinflammatory cytokines and PGE_2 levels are shown. **(A)** Plasma PGE_2 levels from infected mice. **(B–F)** Proinflammatory cytokines from infected mice. IFN-γ **(B)**, TNF-α **(C)**, IL-2 **(D)**, IL-1β **(E)**, and IL-12 **(F)** levels are shown. Plasma from the heparin-treated whole blood of the healthy mice (control), or mice infected by *nahG* or *gfp*-expressing parasites were used for all experiments.

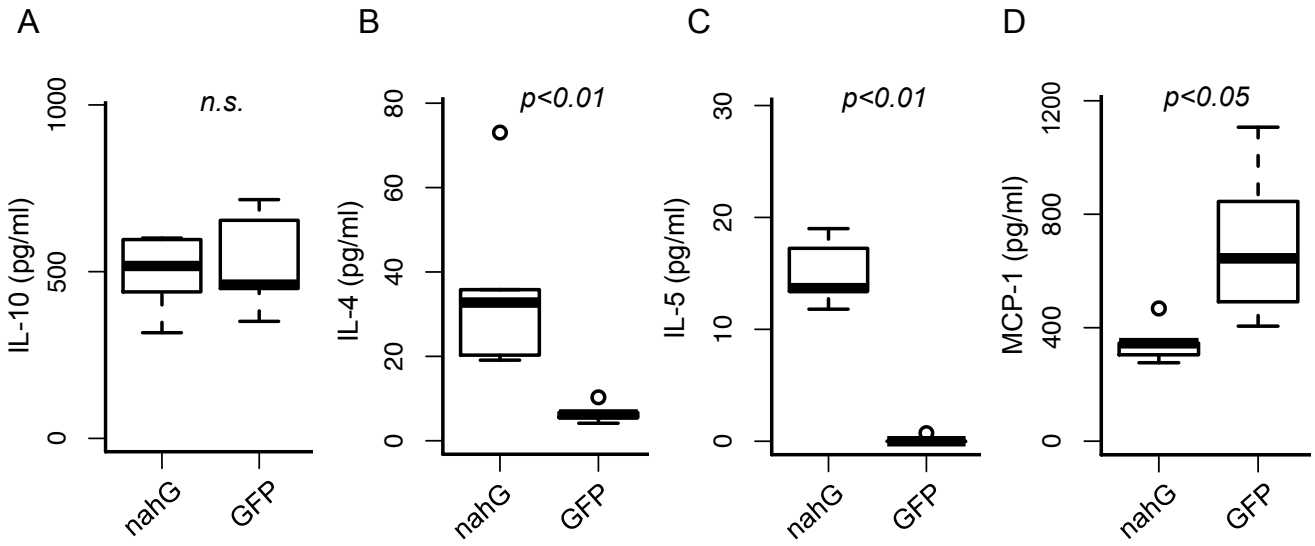

Fig. 23. Plasma anti-inflammatory cytokines and chemokines from infected mice. **(A-C)** Anti-inflammatory cytokines from infected mice. IL-10 **(A)**, IL-4 **(B)**, and IL5 **(C)** levels are shown. **(D)** Inflammatory chemokine MCP-1 levels from infected mice. Plasma from the heparin-treated whole blood of the mice infected by *nahG* or *gfp*-expressing parasites were used for all experiments.